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Seasonal patterns of microcystin producing and non-producing *Planktothrix rubescens* genotypes in a deep prealpine lake

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Abstract

The filamentous cyanobacterium *Planktothrix rubescens* produces secondary metabolites called microcystins (MC) that are potent toxins for most eukaryotes, including zooplankton grazers, cattle and humans. *P. rubescens* occurs in many deep and thermally stratified lakes throughout Europe. In Lake Zurich (Switzerland), it re-appeared in the 1970's concomitant with decreasing eutrophication. Since then, *P. rubescens* has become the dominant species in this major drinking water reservoir, where it forms massive metalimnetic blooms during late summer. These cyanobacteria harbor subpopulations of non-MC producers, but little is known about the environmental factors affecting the success of such genotypes. The non-MC-producing subpopulation of *P. rubescens* was studied using a quantitative real-time PCR (qPCR) assay on the MC synthetase (mcy) gene cluster that targets a deletion on the mcyH and mcyA genes, which inactivates MC biosynthesis. Two complementary qPCR assays were used to assess the total population abundance (based on the 16S rDNA gene) and the mcy gene copy number (based on a conserved region in the adenylation domain of the mcyB gene). The objective was to evaluate the seasonal patterns of the share of non-MC-producing filaments in the total *P. rubescens* population. The mcyHA mutants were present in low proportions (up to 14%) throughout the year. Their highest relative abundances occurred during the winter mixis, when total concentrations of *P. rubescens* were minimal. The MC deficient mutants seemed to better survive in sparse populations, possibly because of lower grazing pressure and a consequently reduced need for MC-mediated protection. Alternatively, the mutants might cope better with the sub-optimal, stressful pressure and light conditions during the winter mixis. Altogether, our results suggest that subtle trade-offs might seasonally determine the proportions of non-MC producers within *P. rubescens* populations.

Keywords

Planktothrix rubescens; Cyanobacteria; Microcystin; mcy gene cluster; Lake Zurich

1. Introduction

The filamentous toxic cyanobacterium *Planktothrix rubescens* is frequently found in deep and thermally stratified lakes. In Lake Zurich, *P. rubescens* recurs every summer and fall, and dominates the autotrophic biomass (Micheletti et al., 1998; Bossard et al., 2001; Posch et al., 2012). *P. rubescens* produces microcystins (MC), which are cyclic heptapeptides that are toxic to most eukaryotes (Blom et al., 2001; 2006); it thus poses a serious health threat to humans and animals. MC are synthesized non-ribosomally by a large enzyme complex encoded on the microcystin synthetase gene cluster (mcy) (Dittmann et al., 1997). The biosynthesis of MC can be inactivated by mutations such as insertions of a transposable element or deletions affecting one or two genes in the cluster (Christiansen et al., 2006; 2008). Experimental studies on the effects of cyanotoxins usually focus on MC, and genotypes that do not produce MC are considered non-toxic (Tillmanns et al., 2008). However, it might be more precise to classify these genotypes as non-MC producers rather than non-toxic because *P. rubescens* is known to synthesize several other oligopeptides toxic to grazers (Blom et al., 2003; Baumann et al., 2007; Kohler et al., 2013).

The co-occurrence of MC-producing and non-producing *Planktothrix* genotypes has been observed several times (Kurmayer et al., 2004; Briand et al., 2008a; Ostermaier and Kurmayer, 2009). While seasonal patterns in the succession of the MC-producing genotypes were suggested by some authors (Janse et al., 2004; Kardinaal et al., 2007a; Davis et al., 2009; Bozarth et al., 2010), other studies showed no positive selection for non-MC-producing *Planktothrix* genotypes during the transition from pre-bloom to bloom conditions (Ostermaier and Kurmayer, 2009; Ostermaier et al., 2012). So far, genetic studies on *P. rubescens* populations in Lake Zurich were confined to the upper 20 meters of the water column whereas the deeper areas, where most *P. rubescens* filaments persist during the 4-month mixis period, have been neglected. As a result, factors that might favor the occurrence of non-MC-producing genotypes remain unclear.

Quantitative real-time PCR (qPCR) is increasingly applied for the enumeration of toxin-producing cyanobacteria in the environment. It is considered an important potential early-warning tool (e.g., Galluzzi, 2012) because it is extremely sensitive, specific. Moreover, it provides estimates of the quantity of a targeted gene sequence, which is a trustworthy proxy for organism abundance. In this study, available qPCR assays targeting mcy genes (Ostermaier and Kurmayer, 2009) were applied on samples collected from the surface down to the winter mixed layer (≤ 100 m) over a period of a year. The objective was to distinguish between MC-producing and non-producing genotypes in order to assess the influence of environmental factors on their respective proportions in the entire *P. rubescens* population. Assuming that genotypes are not selected because of a function directly related to MC but rather because of a fitness advantage (Neilan et al., 2013), one can hypothesized that non-MC-producing genotypes might have a selective benefit under sub-optimal conditions, specifically during pre-bloom and post bloom conditions.

2. Materials and methods

2.1. Study site and sample collection

Lake Zurich is located south of the city of Zurich at 406 m above the sea level on the Swiss Plateau, at the northern edge of the Swiss Alps. This deep (136 m) and large (68 km^2) lake is mesotrophic (10–20 µg L^{-1} total phosphorus; Bossard et al., 2001) and monomictic because it mixes from surface to bottom once a year, between March and April (Posch et al., 2012). This complete water turnover (holomixis) brings nutrients to the illuminated surface waters where photosynthesis takes place, and replenishes oxygen in deep waters. For the period from 1972 to 1990, oxygen concentrations at the bottom of the lake (120–136 m) were highest in April (Posch et al., 2012). The isoline where the maximal oxygen concentration is found, i.e. the median value of 6 mg O_2 L^{-1} for the 18-year period, can be used as a proxy for mixis depth. Data of the last 40 years show an increasing trend of partial mixis caused by lake warming (Posch et al., 2012).

Routine sampling for environmental parameters was carried out every other week from January 2012 to May 2013. A total of 35 profiles were recorded using an YSI-6600 multi-parameter

probe (YSI Incorporated). Parameters were measured from surface to 120 m with sensors for depth (m), temperature (ºC), dissolved oxygen concentration (mg L^{-1}) and saturation level (%), chlorophyll fluorescence (a proxy for the total phytoplankton biomass expressed in relative fluorescence unit, RFU), and for phycoerythrin fluorescence (a proxy for *P. rubescens* biomass, also in RFU). All sensors were calibrated according to the manufacturer's recommendations prior to sampling. Biomass data of the main zooplankton grazers (i.e., daphnids and copepods) in the upper 20 m of the water column were provided by the Zurich Water Supply Company.

Sampling for DNA collection was conducted twice a week during the spring bloom period and until the onset of the thermal stratification. For the remaining year, samples were collected twice a month. From February 1, 2012 to March 15, 2013, a total of 129 samples were obtained above the deepest point of the lake (47°18'25.24"N, 8°34'37.8"E). Samples were collected in duplicate to account for the variability that may have been caused by error during sampling, filtration, and lysate preparation. The year was divided in two parts: the mixing period, i.e., winter and spring, when the temperature was homogeneous from surface to bottom allowing for wind-induced mixing of the lake (Fig. 1A); and the stratification period, i.e., summer and fall when the water column was thermally stratified in three stable layers (epi-, meta- and hypolimnion) that were resistant to wind mixing (Fig. 1B). Environmental parameters were measured using the YSI-6600 probe as described above. Additionally, a spherical quantum sensor (LI-COR) was installed on the probe to measure the in situ photosynthetically active radiation (PAR; µmol m^{-2} s^{-1}). Samples were collected at different depths selected based on the vertical profiles of algal pigments recorded with a fluoroprobe (bbe Moldaenke GmbH). The probe was calibrated to distinguish between diatoms, chlorophytes, cryptophytes, and *P. rubescens* according to optical fingerprints of their pigments. Algal groups were measured in chlorophyll *a* equivalents (µg chl *a* L^{-1}). Cryptophytes and chlorophytes never induced a clear signal above the sensor detection level. The criterion for determining the sampling depths was the maximal chl *a* concentration allocated to *P. rubescens*, which was defined as the *Planktothrix* layer (P_L). Samples taken above and below P_L were assigned

to the surface (S_L) and bottom layers (B_L), respectively. During the mixing period, *P. rubescens* filaments were gradually entrained from surface to depth, which made it difficult at times to discern a P_L in such a quite homogeneous water column (Fig. 1A). In those cases, samples were taken at fixed depths where *P. rubescens* was present. On three occasions in fall 2012, samples were collected at the lake surface where *P. rubescens* filaments accumulated. Such accumulation of *Planktothrix* filaments may be triggered by strong winds, creating red surface scums (Walsby et al., 2005).

The effect of potential unpredictable fluctuations in the sensor readings (i.e., the random error) was minimized by data normalization over the entire vertical profile to discrete depth levels (0.25-m intervals) by linear interpolation (Zeder, 2005). Since water samples were collected with a 5-liter sampler (Uwitec) with a height of 0.66 m, the sensor value had to reflect the average characteristics of the water obtained by this device. Thus, the sensor value at a given sampling depth was calculated as the arithmetic mean of three values: the normalized value at the sampling depth itself (which was expected to be in the center of the water sampler), the normalized values at 0.25 m above, and at 0.25 m below the sampling depth.

2.2. Quantitative real-time PCR

DNA for qPCR analyses was collected by filtering 50–200 ml of water onto 25 mm GF/F filters, which were loosely furled and stored in 2 ml of lysis buffer (100 mM Tris [pH 8], 40 mM EDTA [pH 8], 100 mM NaCl, 1% sodium dodecyl sulfate) at −20 °C until processing within 6 months. The qPCR assays have been previously developed, optimized and tested for specificity (Ostermaier and Kurmayer, 2009) to target the mcy gene cluster of *P. rubescens*. Even though the assays have already been described in detail by these authors, the *M*inimum *I*nformation for *P*ublication of *Q*uantitative real-time PCR *E*xperiments (MIQE; Bustin et al., 2009) is nevertheless listed here, including slight modifications to the original protocol.

The assays targeted: (1) the 16S rDNA gene of *Planktothrix* spp. to quantify the total population; (2) the mcyBA1 region, a conserved region in the adenylation domain of the mcyB

gene to quantify all genotypes containing the mcy gene cluster; and (3) the mcyHA mutation, a deletion within mcyH and mcyA resulting in the inactivation of MC biosynthesis, to quantify this non-MC-producing genotype. This workflow should target all *P. rubescens* filaments on the grounds that the red-pigmented, phycoerythrin-rich lineage occurring in deep stratified lakes usually only consists of genotypes that contain the mcy gene cluster (Kurmayer et al., 2004). The genotype carrying the mcyHA mutation was chosen over other inactive mcy genotypes because it was previously found to be significantly more prevalent in *P. rubescens* populations of twelve alpine lakes (Ostermaier and Kurmayer, 2009). The oligonucleotides for these three assays were synthesized and purified by high-performance liquid chromatography (HPLC) by Biomers.net and sequences are listed in Table 1. The hydrolysis probes were labeled with a fluorescent reporter dye that was covalently attached to the 5′ end (FAM, 6-carboxyfluorescein) and a fluorescent quencher dye attached to the 3′ end (TAMRA, 6-carboxytetramethylrhodamine).

The specificity of primers and probes of each assay was re-examined against the GenBank nucleotide collection on 18 Feb 2014 using the BLASTN function that was optimized for highly similar sequences and adjusted for short input sequences (BLASTN 2.2.29+; Altschul et al., 1997). Only hits that returned the most significant expect value were considered for detailed analysis. Among the 190 significant hits obtained with the 16S rDNA forward primer, up to 89% sequences were “very likely” *Planktothrix.* The reverse primer returned 281 significant hits of which 85% were “very likely” *Planktothrix*, whereas for the TaqMan probe this proportion was 77% of the 218 significant hits. The primers and probe of mcyBA1 region assay returned 49, 54, and 56 significant hits for forward primer, reverse primer and probe, respectively, and they were all associated to the genus *Planktothrix*. Similar results were obtained for the mcyHA assay; very few hits were significant (6 for forward primer, and 19 for both reverse primer and probe), but they were all specific to *Planktothrix* sp.

2.3. DNA extraction and qPCR reaction conditions

The GF/F filters were soaked in lysis buffer for extraction by use of crude lysates as described in Garneau et al. (2013). This procedure is the most appropriate for nucleic acid extraction from microbial cells because there is no loss of genomic DNA (Penna and Galluzzi, 2014). Essentially, cells were lysed during cycles of a thermal treatment followed by a step of physical disruption through vortexing with 0.5-mm zirconia/silica beads. The lysates were stored into 2-ml DNA loBind Tube (Eppendorf) and kept at −20 °C. The lysates were diluted 1:100 to minimize PCR inhibition prior to use in 25-µl qPCR reactions.

Reaction setups were: 5 µl of DNA template, variable concentrations of primers and probes (Table 1), and 1× (final conc.) of master mix. The mix used for the 16S rDNA and mcyBA1 assays was the TaqMan Universal PCR Master Mix (Life Technologies Europe BV). To improve the PCR efficiency of the mcyHA assay, the iTaq Universal Probes Supermix from Bio-Rad was used, and the annealing temperature was set to 59.7 °C. PCR reactions and a blank control were set up in triplicate in 96-well PCR plates sealed with flat cap strips (Bio-Rad Laboratories AG, catalogue #2239441 and #TCS0803, respectively). The thermal cycling conditions were: one initiation step of a 10-min hold at 95 °C to activate the hot-start polymerase, and then 45 cycles of a two-step PCR, consisting of a denaturation phase at 95 °C for 15 s, followed by the annealing/extension phase for 1 min at the temperature indicated in Table 1. Thermal cycling and real-time data collection at the extension step were performed using a CFX96^{TM} Real-Time PCR Detection System (Bio-Rad Laboratories AG). The qPCR quantification cycles (C_q) and their associated standard deviations were determined from a single threshold that was automatically calculated using the CFX Manager software.

2.4. Calibration of the qPCR assays for quantification

The calibration curves for the 16S rDNA and mcyBA1 assays were made using a culture of strain A7 isolated from Lake Zurich in 1993 by A. E. Walsby. *Planktothrix* cultures were used to overcome the problem of varying genome copy number per cell in cyanobacteria (Becker et al.,

2002), assuming that copy numbers in cells behave similarly in both culture and in natural conditions. A mcy mutant *P. rubescens* culture (strain 62) was used for the calibration of the mcyHA assay. Strain 62 was isolated in 2001 from surface water of Irrsee (Austria), and was kindly provided by R. Kurmayer. Both cultures were grown in Erlenmeyer flasks on a medium as described by Jüttner et al. (1983) at 20 °C and under constant light at 5 µmol m^{-2} s^{-1}.

Standards for the calibration curves were prepared from four 1:10 serial dilutions of a known concentration of growing *P. rubescens* cultures (strains A7 and 62). Dilutions were made with 0.2-µm filtered water. A serial dilution of natural (unfiltered) water was also analyzed to ensure that natural constituents (e.g., particles or non-target organisms) would not interfere with the detection of *P. rubescens*. Each standard was filtered on triplicate GF/F filters, which were processed as described before to generate lysates. To determine the concentration in the cultures, *P. rubescens* filaments were fixed with formaldehyde (final conc. 2%) and filtered onto black polycarbonate filters (0.22-µm pore size, Osmonics). Filament abundances and lengths within the original cultures were determined using autofluorescence microscopy under green light excitation (Zeiss filter set 43) with a Zeiss AxioImager.Z1 microscope at a magnification of ×10. The microscope was equipped with a very sensitive charge-coupled device camera (AxioCam MRm, 12 bit grayscale, Zeiss). A composite image (jpg files of 10133 × 9466 pixels) was produced for each microscopic slide by the software module MosaiX of the image analysis software Axio Vision 4.6.3 (Zeiss). Between 100 and 300 filaments were counted and measured by image analysis (Zeder et al., 2010). The biovolume was then calculated as in Van den Wyngaert et al. (2011). Total biovolume in mm^3 L^{-1} was calculated by multiplying the number of filaments by the mean filament volume.

A calibration curve was run in triplicate with every qPCR assay in parallel with a triplicate set of unknown samples to facilitate the conversion of C_q values of the unknowns into abundances. The triplicate no-template controls that were also included in each qPCR plate did not returned detectable signals. The qPCR amplification efficiency (*E*) was calculated as:

$$E = 100 \times 10^{\left(\frac{-1}{m}\right)} - 1 \quad (1)$$

where m is the slope of the calibration curve. Acceptable E values for assay validation vary between 90% and 110% (Invitrogen, 2008).

There was a highly significant regression between two independent estimates of *Planktothrix* abundances, i.e., the biovolumes estimated with the 16S rDNA assay and the *Planktothrix* concentrations measured with the fluoroprobe (Fig. 2), indicating that our approach closely matched the in situ distribution. Outlier qPCR results were detected using an equation that was previously determined for Lake Zurich:

$$Chla = 3.92 \times P.rub + 2.73 \qquad n = 144,\ r^2 = 0.94 \quad (2)$$

where *Chla* is chlorophyll *a* concentrations in µg L^{-1} determined after acetone extraction and *P.rub* is *P. rubescens* biovolume in mm^3 L^{-1} based on microscopic counts (Van den Wyngaert et al., 2011). Here, *P.rub* was replaced by biovolumes determined with the 16S rDNA assay and *Chla* by the values measured with the fluoroprobe at the corresponding depths. Seven of the 258 biological duplicates were discarded because they were highly inconsistent with the fluoroprobe values and thus were not analyzed with the two other qPCR assays.

2.5. Statistical analysis

Statistical analyses were performed using the R software (R Core Team, 2013). Linear regression models were computed using the lm function to determine the qPCR calibration curves, and to evaluate the relationship between the mcy genotypes and the total population. The assumptions of the simple linear regression were reasonably fulfilled for the biovolumes data. However, the Shapiro-Wilk normality test indicated that most of the environmental data were not following a normal distribution, thus non-parametric tests were employed. Medians and quartile ranges (Q1 and Q3) were used to describe the data.

Spearman's rank correlation coefficient (r_S) at the level of significance $p < 0.05$ were employed to quantify the association between two variables, namely the biovolumes and the environmental parameters. The different seasons were compared with Kruskal-Wallis analyses (KW) and the two periods (mixing and stratification) with Mann-Whitney analysis (MW). The null hypothesis assuming that mean ranks of samples are the same for all the groups was rejected when $p < 0.05$. Multiple comparisons post-hoc tests were run using the kruskalmc function in the pgirmess package (Giraudoux, 2014) to determine which sampling depths, seasons or periods differed from each other.

3. Results

3.1. Calibration, efficiency and specificity of the qPCR approaches

The calibration curves for the three qPCR assays were linear regressions constructed by plotting the log of the starting quantity of template in each qPCR reaction against the C_q obtained during the amplification of each template (Table 2). The calibration curve for 16S rDNA and mcyBA1 was linear for a concentration of 3.75 to 3.75 $\times 10^{-3}$ mm^3 L^{-1}, which corresponded to 2.34 $\times 10^{-5}$ to 2.34 $\times 10^{-8}$ mm^3 of filaments per qPCR reaction. The calibration curve for mcyHA was linear for a concentration of 1.46 $\times 10^{1}$ to 1.46 $\times 10^{-2}$ mm^3 L^{-1} (or 9.10 $\times 10^{-5}$ to 9.10 $\times 10^{-8}$ mm^3 per qPCR reaction). The lowest concentrations were detected before C_q equaled 36. The standard deviations of C_q at the limit of detection were ≤ 0.7 (Table 2). The *E* values were within the acceptable range for assay validation (Invitrogen, 2008) for both the 16S rDNA and mcyHA assays (Table 2). The *E* value for mcyBA1 was slightly above this range, but the genes were amplified with comparable efficiencies, allowing for comparison.

3.2. Accuracy, repeatability and reproducibility of the qPCR approaches

The accuracy of the 16S rDNA analysis was confirmed by comparing qPCR results to microscopic slides counted in triplicates for eleven lake water samples. The two quantification methods gave highly correlated biovolume values (slope $m = 0.82$, $r^2 = 0.93$). The average coefficient of variation on the biovolumes between the duplicate filters was less than 20% for all the

three assays. All assays combined (129 samples × 3 assays = 387 reactions), there were 16 instances where the variability was between 30-40%. The variation between runs for a given sample, was on average 26% (n = 11), 27% (n = 9) and 33% (n = 6) for the 16S rDNA, mcyBA1 and mcyHA assays, respectively.

3.3. Biovolumes of P. rubescens*, mcyBA1 and mcyHA genotypes in Lake Zurich*

Lake Zurich was thermally stratified in the summer and fall (Figs 1B, 3A), allowing *P. rubescens* to accumulate in the metalimnion (Figs 1B, 3C). During winter however, the thermal stratification eroded (Figs 1A, 3A), enabling the water column to mix and disperse *P. rubescens* filaments down to the hypolimnetic zone (Figs 1A, 3C). Holomixis occurred in May 2012, when oxygen concentrations reached 6 mg L^{-1} at 120 m (Fig. 3B). The 6 mg O_2 L^{-1} isoline between 10 and 60 m during fall represents the typical oxygen minimum found below the *Planktothrix* layer (Fig. 3B). This is likely caused by respiration of *P. rubescens* filaments when they are entrained below the compensation depth (Garneau et al., 2013). Grazing pressure on phytoplankton was highest in May 2012, as inferred by the biomasses of the two main grazer groups, daphnids and copepods (Fig. 3D).

The total *P. rubescens* population, quantified from 16S rDNA, showed the expected seasonal variations (Fig. 4A). Altogether, values spanned from 0 to 5.9 mm^3 L^{-1} and had a median of 0.68 mm^3 L^{-1}, n = 126. Biovolumes in winter were significantly different from the ones in spring and summer; similarly, the biovolumes found in fall were different from the spring-summer period (KW, $p < 0.05$; Fig. 4A). The highest values occurred in fall and the lowest in summer (Fig. 4A).

The biovolume of the mcyBA1 genotype stayed rather stable around an annual median value of 0.79 mm^3 L^{-1} (n = 126, Fig. 4B), and its ratio to the total *P. rubescens* population did not exhibit a seasonal pattern. The biovolumes of *P. rubescens* as deduced from quantifying the mcyBA1 genotype were similar to those determined by 16S rDNA. The relationship between the two variables was linear and described as:

$$y = 1.1x + 0.11 \qquad n = 126,\ r^2 = 0.95,\ p < 0.001 \qquad (3)$$

where x is the biovolume estimated by 16S rDNA and y is the mcyBA1 biovolume. Slight divergence between the two assays may be due to the small difference in the amplification efficiencies (E).

The inactive mcyHA genotype was detected in 75% of the samples, and contributed from 0.8 to 14% (median of 3.3%) to the total 16S rDNA biovolume. Biovolumes of the inactive genotype displayed the same seasonal trend as the total *P. rubescens* abundances, as detected by the 16S rDNA qPCR assay (Fig. 5A). However, the ratio of mcyHA genotype to the total population, hereafter referred to as mcyHA:16S, showed a markedly different seasonal pattern (Fig. 5C). Accordingly, mcyHA:16S ratios in winter were significantly different from all other seasons (KW, $p < 0.05$; Fig. 5C).

Total *P. rubescens* biovolumes in samples collected in fall during three distinct events of surface accumulation were above the median value (Table 3). The mcyBA1:16S ratios were close to 1 (median 1.2), but tended to be slightly higher than values observed in all other samples. The inactive genotype mcyHA was also detected in these samples, in proportions of up to 3.8% of total biovolume (Table 3).

Guided by the ecology of *P. rubescens,* the data were divided in two subsets: the mixing (winter-spring) and stratification (summer-fall) periods (Fig. 5B, D). The biovolumes of *P. rubescens*, as well as of the mcyBA1 and mcyHA (Fig. 5B) genotypes were not different between the two periods, but the mcyHA:16S ratios were (Fig. 5D; MW $p < 0.0005$); this means that the relative importance of the inactive mcyHA genotype was higher during the winter mixis (median = 3.0 %, $n = 98$) than during the summer stratification (1.3%, $n = 18$). By contrast, the mcyBA1:16S ratio did not show any difference between the two periods. During the stratification period ($n = 28$), the total *P. rubescens* biovolumes (as deduced from 16S rDNA) were statistically indistinguishable between depth layers although higher values were found in the P_L. Median values

and quartile ranges (Q1 and Q3) in $mm^3\ L^{-1}$ were: 0.06 (0.02 and 2.25) for S_L; 3.31 (1.03 and 4.56) for P_L; and 0.27 (0.04 and 1.10) for and B_L. Similar results were obtained for the biovolumes of the two mcy genotypes. The proportions of mcyBA1 and of mcyHA mutants in the total *P. rubescens* population did not change with depth layer.

3.4. Biovolumes and environmental parameters

Many variables were co-correlated and thus delivered similar information, such as total chlorophyll *a* and *P. rubescens* proxy (i.e., 16S rDNA biovolume, fluoroprobe concentration of *Planktothrix*), and oxygen saturation level and water temperature. Nonetheless, meaningful moderate (0.3 to 0.5) and inverse correlations were found between air temperature and total *P. rubescens* biovolumes, as well as with the mcyBA1 and mcyHA genotypes (Table 4). The direct correlation with air temperature reflects seasonal variations, whereas the absence of a correlation with water temperature likely results from the sampling strategy: water temperatures were determined for the sampling depth where *P. rubescens* filaments were collected, and are not the mean temperature for the entire water column. During the stratification period, filaments were collected in the thermocline, which shows sharp gradients in temperature.

A covariation with air temperature was also found for the mcyHA:16S ratios, but not for the ratios of mcyBA1:16S (Table 4). An inverse correlation was found between mcyHA:16S and water temperature (Table 4). The mcyHA:16S ratios also co-varied positively with the depth of the euphotic zone, and negatively with the in situ irradiance levels (Table 4).

4. Discussion

Seasonality of the inactive microcystin genotype

The study presents quantitative information about *P. rubescens* population genetics in Lake Zurich together with abiotic parameters over an annual cycle to better understand possible environmental selection for a particular genotype of non-MC producers. The sampling scheme was adapted according to seasons, and water was specifically collected from depths where *P. rubescens*

was present, i.e., in the epi- and metalimnion in summer and fall, and including deeper water layers during the winter mixis. As a result, the survey comprehensively documents the seasonal variability of the relative abundances of the mcyHA and mcyBA1 mutant genotypes within the total *Planktothrix* population.

As reported previously (Ostermaier and Kurmayer, 2009), biovolumes of inactive mcyHA genotype was correlated to the biovolumes of the total population (as determined by 16S rDNA), and both were most abundant in fall and winter (Figs 4A and 5A). The ratio of the mcyBA1 genotype to 16S rDNA was stable throughout the year and close to 1 (Fig. 4B), as also observed in Kurmayer et al. (2011). These results are consistent with prior findings that *P. rubescens* populations were only composed of genotypes that carry the mcy gene cluster (Kurmayer et al., 2004; Ostermaier and Kurmayer, 2010). The ratio of inactive mcyHA genotype to 16S rDNA in winter was significantly different from all other seasons (Fig. 5C). In sparse population, such as during the vernal mixing, the proportion of the non-MC-producing genotype in the total population was higher (Fig. 5D).

The *Planktothrix* biovolumes (median 0.68, mean 1.3 $mm^3 L^{-1}$) in 2012-2013 compared well with the annual mean of 1.6 $mm^3 L^{-1}$ determined for a 29-year period (Ostermaier et al., 2012). While the maximal value in 2012-2013 was somewhat higher than the long-term one (5.9 vs. 4.2 $mm^3 L^{-1}$), it was confirmed by in situ measurements with the fluoroprobe. Higher values also agree with the ongoing trend of increasing *Planktothrix* biomass in Lake Zurich caused by weaker vernal mixis due to lake warming (Posch et al., 2012). The proportions of the mcyHA deletion genotype in Lake Zurich in our study (0.8 to 14%, mean of 3.6%) were also in good agreement with previous observations from a single time point (< 6.5%; Ostermaier and Kurmayer, 2009) and the above-mentioned long-term survey (0.3 to 7.2%, annual average 3.3%; Ostermaier et al., 2012). Slightly higher proportions of the mcyHA biovolumes in our study may in part be due to differences in qPCR amplification efficiency, as suggested by mean efficiency values of 85% (Ostermaier et al., 2012) vs. 107% (Table 2). It is important to note that only one non-MC-producing genotype was

considered here, i.e., the mcyHA deletion, which is the most abundant mutant (Ostermaier and Kurmayer, 2009), as well as the only one that was consistently detected in Lake Zurich from 1980 to 2008 (Ostermaier et al., 2012).

Our findings nevertheless contrast with the above long-term study that reported a seasonally rather stable proportion of the non-MC-producing genotypes (Ostermaier et al., 2012). This difference between the two studies may be due to our adaptive sampling strategy. In the long-term survey, samples were collected at four discrete depths from the upper water body (0, 5, 10 and 20 m). This sampling was not entirely representative of the winter mixis period from January to April, during which more than 50% of the total *Planktothrix* biomass is located below 20 m (Posch et al., 2012). By contrast, our study comprised a larger variety of conditions experienced by *P. rubescens,* including the layer of its distribution optimum and its transit towards deep waters where light, nutrient concentration and hydrostatic pressure change constantly.

Does microcystin production affect fitness?

Microbial populations frequently harbor genotypic subpopulations that have lost the ability to produce particular secondary metabolites, such as MC in *Planktothrix* (Kurmayer et al., 2004; Briand et al., 2008a; Ostermaier and Kurmayer, 2009). In the context of the so-called 'public goods' concept (e.g., siderophores), such subpopulations are frequently referred to as 'cheaters' (Velicer 2003). While MC are not typically released from intact cells and would thus qualify as 'private goods', their deleterious effect on predators is arguably only effective if a large share of the population maintains production. During the growth period of the studied cyanobacteria, there are pronounced changes in environmental conditions; the accumulation of dense biomass increases water turbidity and reduces light availability (Walsby et al., 2001; Garneau et al., 2013), gradual resources consumption significantly decreases the stocks of inorganic carbon (Ibelings and Maberly, 1998) and nutrients (Kardinaal and Visser, 2005), and grazing pressure intensifies (Fig. 3D). Thus, there might be selective forces in favor of, or against, MC-producing genotypes.

Prior studies addressing the competitive potential of MC-producing and non-MC-producing genotypes in relation to seasonal changes have reached conflicting conclusions. While MC-producing genotypes of *Microcystis* spp. grew better than non-producing ones with high nutrient concentrations in experiments (Vézie et al., 2002) and in the field (Yoshida et al., 2007), other experimental studies demonstrated that MC-producing genotypes had a higher fitness advantage under growth-limiting conditions (e.g., low light intensity, temperature, inorganic carbon and nutrient concentrations). This was shown for both *Microcystis aeruginosa* (Leblanc Renaud et al., 2011; Van de Waal et al., 2011) and *P. agardhii* (Briand et al., 2008b). It has been argued that even though the production of MC is costly, its advantages under stress conditions may compensate for the physiological costs. In agreement with this reasoning it was also found that non-toxic *M. aeruginosa* genotypes were selected under optimal growth conditions in the field (Briand et al., 2009). These findings are in contradiction with the here observed seasonal pattern of the relative abundances of a non-MC-producing *P. rubescens* genotype (Figs 5C, D), as well as with other field studies showing that non-MC-producing *Microcystis* genotypes dominated under nutrient- (Otten et al., 2012) or light-limiting (Kardinaal et al., 2007b, Van de Waal et al., 2011) conditions, and during the decline of the bloom (Janse et al., 2004; Kardinaal et al., 2007a; Bozarth et al., 2010).

The contrasting conclusions about the potential selective advantages of MC-producing or non-producing genotypes under specific environmental conditions may in fact result from a co-selection of phenotypic differences other than MC production (Neilan et al., 2013). Most studies addressing this issue have focused on *M. aeruginosa*, *P. agardhii* and *P. rubescens,* which all occupy distinct ecological niches. While the two former are usually found in shallow, eutrophic lakes and are adapted to higher temperatures and elevated light exposure (Paerl et al., 2001; Oberhaus et al., 2007), *P. rubescens* inhabits the metalimnion of deep oligo- and mesotrophic lakes where it thrives under low light and cooler temperature (Davis and Walsby, 2002; Oberhaus et al., 2007). During lake mixis, *P. rubescens* is entrained at great depths (> 100 m in Lake Zurich), but its

gas vesicles may largely withstand the elevated hydrostatic pressure between 67 and 99 m (Bright and Walsby, 1999).

Hence, the three species experience different conditions as stressful, and the selection for non-MC-producing genotypes is possibly not driven by the same environmental factors for *P. rubescens* than for *P. agardhii* and *M. aeruginosa*. The reasons for an increased proportion of non-MC-producing *P. rubescens* genotypes in Lake Zurich during the stressful period of the vernal mixing are presently unclear. Several studies have been unsuccessful in finding obviously reduced fitness of non-MC-producing genotypes when compared to MC-containing wild-type cells, both in laboratory experiments and natural conditions (see review of Sivonen and Börner, 2008). In the following, possible explanations for our observation are discussed in the context of the alleged ecological roles of MC in *P. rubescens.*

Microcystins as defensive mechanism

Toxicity experiments have demonstrated that MC can act as a deterrent for grazers (Kurmayer and Jüttner, 1999), suggesting that MC-deficient filaments undergo a more severe grazing pressure than the MC-containing cells. Grazing is generally more important during the phytoplankton growth season (Fig. 3D), when the water column is stratified and *P. rubescens* accumulates into a dense layer within the metalimnion (Figs 1B, 3C). During the winter mixis however, *P. rubescens* density is low because the filaments are dispersed throughout the water column (Figs 1A, 3C). Since there is also little zooplankton during this period (Fig. 3D), grazing pressure on *P. rubescens* is consequently low. It is conceivable that there might be a reduced need for the protection provided by MC in sparse *P. rubescens* population, which could be a reason for the increased fraction of MC-lacking filaments during winter mixis.

It is also plausible that the presumed disadvantage of the loss of MC production by *Planktothrix* may be functionally compensated by the production of other unknown bioactive peptides (Kurmayer and Jüttner, 1999; Hulot et al., 2012). This is particularly relevant for *P. rubescens*, which is known to produce various toxic secondary metabolites (Blom et al., 2003;

Baumann et al., 2007). Recently, a toxic peptide of the class of aeruginosins that is characterized by chlorine and sulfate moieties was isolated from a *P. rubescens* strain with inactive mcyHA, and its toxicity to crustacean grazers has been confirmed in tests with the freshwater crustacean *Thamnocephalus platyurus* (Kohler et al., 2013). Thus, the inactive mcyHA genotype detected by qPCR during the field survey of 2012-2013 may in fact have produced other toxic and/or grazer deterrent compounds instead of MC. It is unknown whether such an alternate compound would cost less to synthesize.

Microcystin production and light

The grazer-deterrent hypothesis of MC production has been challenged by comparative phylogenetic analysis: MC synthetase genes code for an ancient secondary metabolic pathway in many cyanobacteria, and have probably appeared before the metazoans (Rantala et al., 2004). Moreover, there is growing evidence of an intracellular role for MC as protectant against oxidative stress in *M. aeruginosa* (Kaebernick et al., 2000; Zilliges et al., 2011) and *P. agardhii* (Tonk et al., 2005). However, a central function of MC as an oxidative protectant cannot be readily envisaged for a metalimnetic species such as *P. rubescens* because it is rarely exposed to the high surface irradiance that is experienced by *M. aeruginosa* and *P. agardhii*. Nevertheless, *P. rubescens* filaments are periodically brought to the surface in autumn (Walsby et al., 2005), where they can be exposed to high irradiances. Such surface scums were observed on three occasions, during which the in situ light varied between 93 to 1048 µmol m^{-2} s^{-1} (Table 3). These values are substantially higher than summer irradiance levels in the metalimnion (0.30 to 37; Garneau et al., 2013). Considering the oxidative protection hypothesis, such conditions should have strongly selected for MC-producing genotypes over non-producers. However, mcyHA mutants were detected in appreciable proportions, the highest value of 3.8% being found under the highest irradiance regime (Table 3). It is conceivable that such ‘upwelling’ events might be not of sufficient duration to shift the genotypic ratios of a slowly growing organism such as *P. rubescens*.

Highest proportions of the mcyHA genotype occurred in winter when irradiance is low due to shorter day light period and lower solar angle, which is why an inverse correlation was found between the non-MC-producing genotype and light in Lake Zurich (Table 4). During winter, the overall growth conditions are far from optimal, so that the increased proportion of the non-MC-producing genotype is more likely related to better survival than to faster growth. This may be important for the overall population genetics: non-MC-producing filaments will be recruited for the next growth period in higher proportions than their average annual occurrence, which may help to explain the persistence of the non-MC-producers in the population, despite the above discussed possible fitness disadvantages.

MC genotypes and hydrostatic pressure

The selection for MC-producing or non-producing genotypes may be driven by other environmental characteristics that provide higher ecological fitness to a given genotype. This could be the case for the mcyHA genotype during the winter and early spring periods, where cyanobacterial growth is reduced because of sub-optimal temperature, mixing and high pressure at depth. The winter 2012-2013 was particularly cold, and water turnover reached down to 120 m (Fig. 3B), a depth at which the hydrostatic pressure causes the collapse of the gas vesicles and the loss of filaments to the sediments (Bright and Walsby, 1999). Selection for *P. rubescens* genotypes with stronger gas vesicles that remain buoyant after such a deep mixing has been documented (Walsby et al., 1998). Three distinct classes of gas vesicle genotypes were found in Lake Zurich (Beard et al., 1999; Bright and Walsby, 1999). Only the strongest one (GV3) supports the pressure at 99 m, whereas the two others genotypes collapse at shallower depth, i.e., below 67 m (Bright and Walsby, 1999). However, a greater cost is associated with the production of stronger gas vesicles (Walsby, 1994). In a co-occurring population of *P. rubescens* and *P. agardhii*, the variations in chemotype composition was explained by a possible association between depth regulation by gas vesicles and oligopeptide production, implying that distinct chemotypes would select for different gas vesicle characteristics (Rohrlack et al., 2008). In view of the enhanced survival of both the

mcyHA and the GV3 genotypes during the winter mixing period, it is conceivable that there might be a co-selection of the two loci. However, a comparative analysis of multiple single filaments, e.g. by genome sequencing or multiplex PCR, would be required to substantiate such speculations.

In summary, a seasonal pattern in the proportions of a non-MC-producing *P. rubescens* mutant in the total population could be revealed by a field-sampling scheme specifically adapted to the ecology of this cyanobacterium. Even though its phenotype seemed more resistant to mixing, the mcyHA genotype is unlikely to dominate over the wild-type genotype because the overall conditions during this period are far from optimal for growth. Rather, the mcyHA genotype may survive longer compared to the wild-type, explaining their increased relative abundance. Since increasing winter air temperature leads to weaker mixing and a longer stratified period in deep monomictic lakes (Posch et al., 2012; Wahl and Peeters, 2014), it is conceivable that the mcyHA genotype in Lake Zurich will be negatively affected by global climate change.

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Table 1. Properties of oligonucleotides for the quantification of the *Planktothrix* total population (16S rDNA), the genotypes containing the mcy gene cluster (mcyBA1), and the inactive microcystin genotype (mcyHA) in Lake Zurich. The probes (sequences 5′ → 3′) are labeled with a fluorescent reporter dye attached to the 5' end (FAM, 6-carboxyfluorescein) and a fluorescent quencher to the 3′ end (TAMRA, 6-carboxytetramethylrhodamine).

Target gene	**Forward / reverse primers**	**Hydrolysis probes**	**Concentrations* (nM)**	**Annealing (°C)**	**Amplicon (bp)**
16S rDNA	ATCCAAGTCTGCTGTTAAAGA / CTCTGCCCCTACTACACTCTAG	AAAGGCAGTGGAAACTGGAAG	300/300/250	55	82
mcyBA1	ATTGCCGTTATCTCAAGCGAG / TGCTGAAAAAACTGCTGCATTAA	TTTTTGTGGAGGTGAAGCTCTTTCCTCTGA	900/900/100	60	76
mcyHA	TCTTCTGGACGGTTTTCTAG / CTTTCCGGGTTTGATGT	TACAGAATGGGAAAAAATTACTCAAGAGAA	300/400/250	59.7	71

*Forward primer/reverse primer/probe

Table 2. Calibration curve equations, correlation coefficients (r^2), PCR amplification efficiency (*E*), standard deviation of the quantification cycle (SD C_q) at the limit of detection, number of replicate calibration curves (*n*), and the coefficient of variation on the biovolumes estimated with each assay (CV). The *y* value is the log of the starting quantity of template and the *x* value is the quantification cycle (C_q) of template.

Target gene	**Calibration curve**	**r^2**	***E*** **(%)**	**SD C_q**	***n***	**CV (%)**
16S rDNA	y = 10.93 - 3.10x	0.99	110	0.3	14	10
mcyBA1	y = 13.22 - 2.96x	0.98	118	0.5	14	11
mcyHA	y = 12.06 - 3.17x	0.96	107	0.7	14	22

Table 3. Characteristics of samples collected in fall 2012 during surface accumulation events of *P. rubescens*. Total biovolumes of *P. rubescens* are based on the 16S rDNA genotype. The mcyBA1 (BA1) and mcyHA (HA) genotypes are presented as percentage of the total biovolumes (16S). PAR: in situ photosynthetically active radiation; Water temp: in situ temperature.

Date	PAR ($\mu mol\ m^{-2}\ s^{-1}$)	Water temp (°C)	16S biovolume ($mm^3\ L^{-1}$)	BA1:16S (%)	HA:16S (%)
27 Sept	1048	18	1.5	114	3.8
11 Oct	93	17	2.4	150	1.6
8 Nov	310	11	5.2	93	1.4

Table 4. Spearman correlation coefficients (r_s) with corresponding number of samples (n) for environmental parameters compared to biovolumes of *Planktothrix* genotypes over a year of sampling. Significance levels are $p < 0.05$ (*) or $p < 0.001$ (**). PAR: in situ photosynthetically active radiation; Water T °C: temperature of the water at the sampling depth; Z euph: euphotic zone.

		Air T °C	Water T °C	Z euph	PAR
16S	r_s	**-0.5 ****	-0.1	0.1	-0.2
	n	*121*	*126*	*121*	*100*
mcyBA1	r_s	**-0.5 ****	-0.1	0.1	-0.2
	n	*121*	*126*	*121*	*100*
mcyHA	r_s	**-0.5 ****	0.0	0.2	-0.1
	n	*121*	*126*	*121*	*100*
mcyBA1:16S	r_s	0.01	-0.2	0.2	-0.2
	n	*121*	*126*	*121*	*100*
mcyHA:16S	r_s	**-0.4 ****	**-0.3 ***	**0.4 ****	**-0.3 ***
	n	*121*	*126*	*121*	*100*

Figure legends

Figure 1. Depth profiles of temperature and of the two main phytoplankton groups, i.e., *Planktothrix rubescens* and diatoms (in µg chlorophyll *a* L^{-1}) in Lake Zurich. (A) Mixing period, when *P. rubescens* was entrained down to 100 m, as observed on 16 February, 2012. (B) Stratification period in the upper water column, when a dense accumulation of *P. rubescens* was observed in the metalimnion on 18 September, 2012. Diatoms were detected in the upper 20 m.

Figure 2. Relationship between the 16S rDNA genotype biovolumes estimated by qPCR and the concentrations of *Planktothrix rubescens* measured with a fluoroprobe. The 16S rDNA genotype biovolumes is an estimation of the total population of *Planktothrix* spp. The regression equation and the 95% confidence intervals (dashed lines) are also presented. Chl *a*: chlorophyll *a.*

Figure 3. (A) Temperature, (B) oxygen concentration and (C) *Planktothrix rubescens* concentration from surface to the bottom of Lake Zurich. (D) Biomass of metazooplankton in the first 20 m. The 6 mg L^{-1} of oxygen isoline in (B) is an indicator for holomixis (see text for details). Chl *a*: chlorophyll *a.*

Figure 4. (A) Box-plot of the biovolumes of *Planktothrix rubescens* over a year, grouped by seasons. The asterisks indicate that biovolumes measured for both winter and fall differ from the ones for spring and summer (Kruskal–Wallis analysis, $p < 0.05$). (B) Box-plot of the ratios of the mcyBA1 genotype to the 16S rDNA genotype biovolumes over a year, grouped by seasons. All biovolumes (in mm^3 L^{-1}) were estimated by qPCR performed on water samples ($n = 126$) collected from February 1, 2012, to March 15, 2013. The white circles are outlier values.

Figure 5. (A) Box-plot of the biovolumes of mcyHA genotype over a year, grouped by seasons. The asterisks indicate that biovolumes measured for both winter and fall differ from the ones for spring and summer (Kruskal–Wallis analysis, $p < 0.05$). (B) Box-plot of the biovolumes of mcyHA genotype over a year, grouped by period. (C) Box-plot of the ratios of the mcyHA genotype to the

16S rDNA genotype biovolumes over a year, grouped by seasons. The double asterisk indicates that the ratio estimated for winter differs from the ones for all other seasons (Kruskal–Wallis analysis, $p < 0.05$). (D) Box-plot of the ratios of the mcyHA genotype to the 16S rDNA genotype biovolumes over a year, grouped by period. The asterisk indicates that the ratio estimated for the mixing period differs from the one for the stratification period (Kruskal–Wallis analysis, $p < 0.05$). All biovolumes (in $mm^3\ L^{-1}$) were estimated by qPCR performed on water samples ($n = 126$) collected from February 1, 2010, to March 15, 2013. The white circles are outlier values.

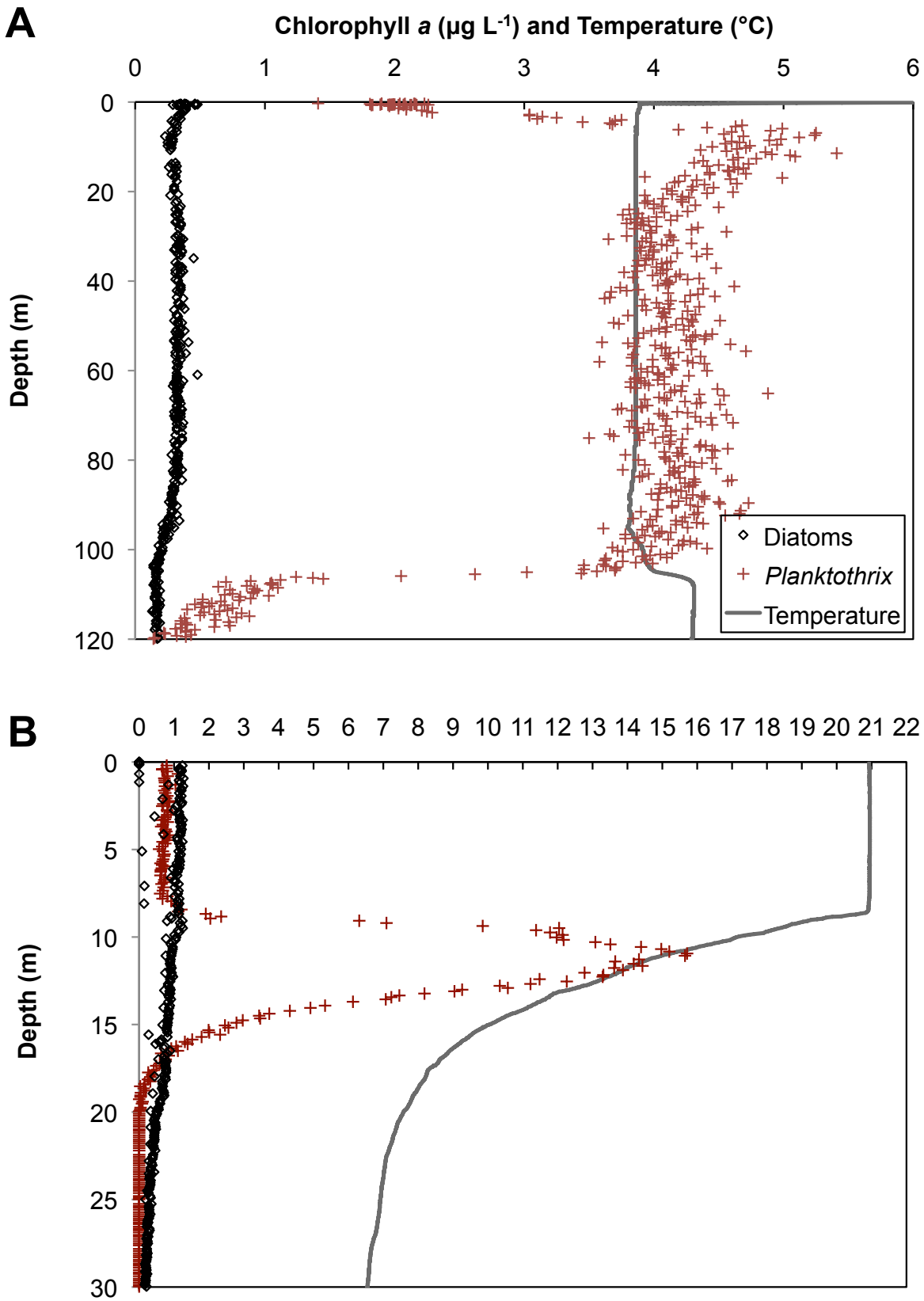

Figure 1. Garneau et al.

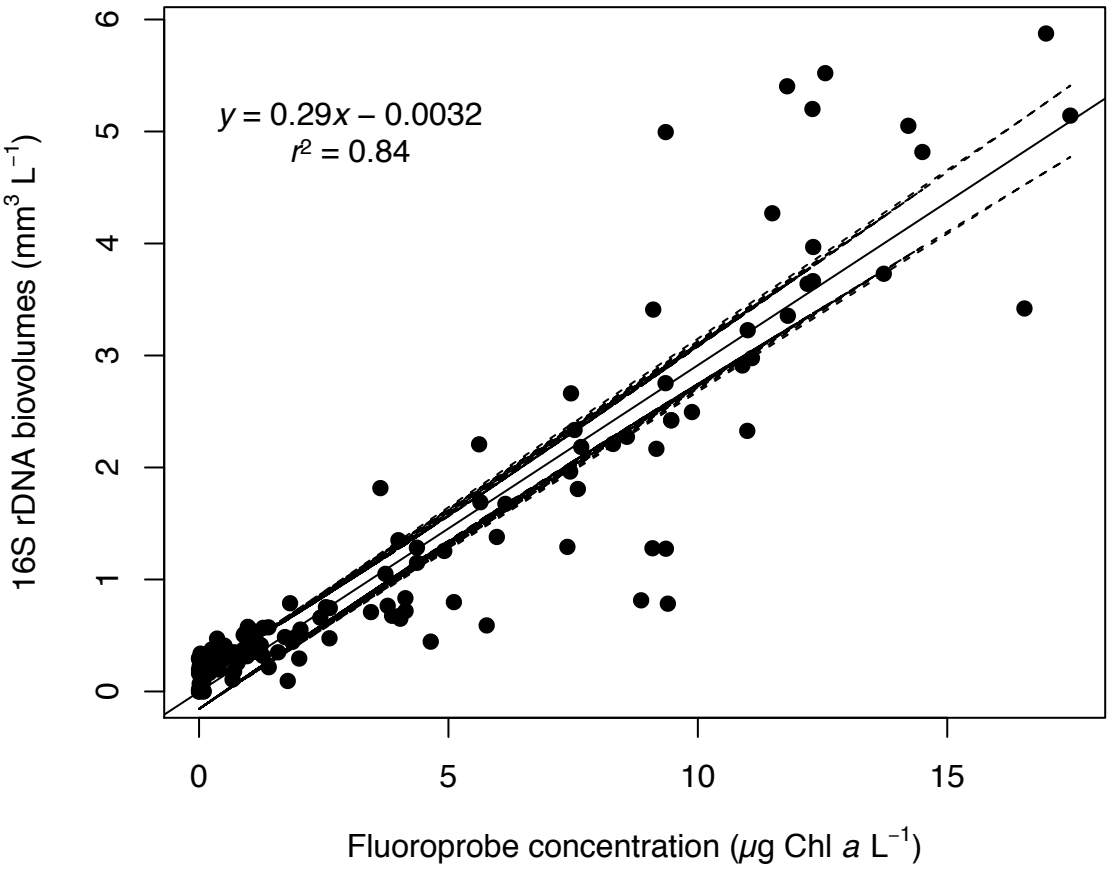

Figure 2. Garneau et al.

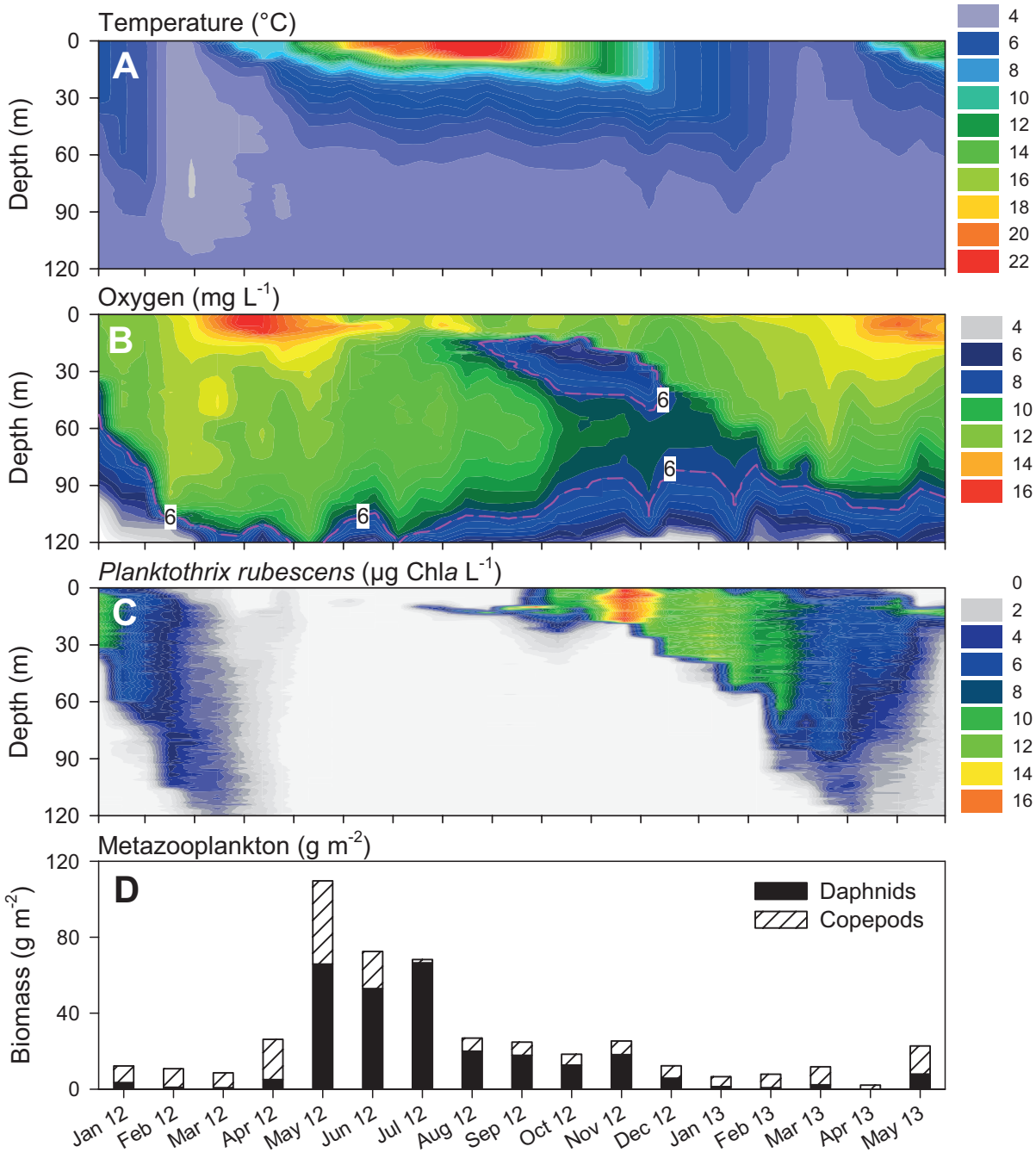

Figure 3. Garneau et al.

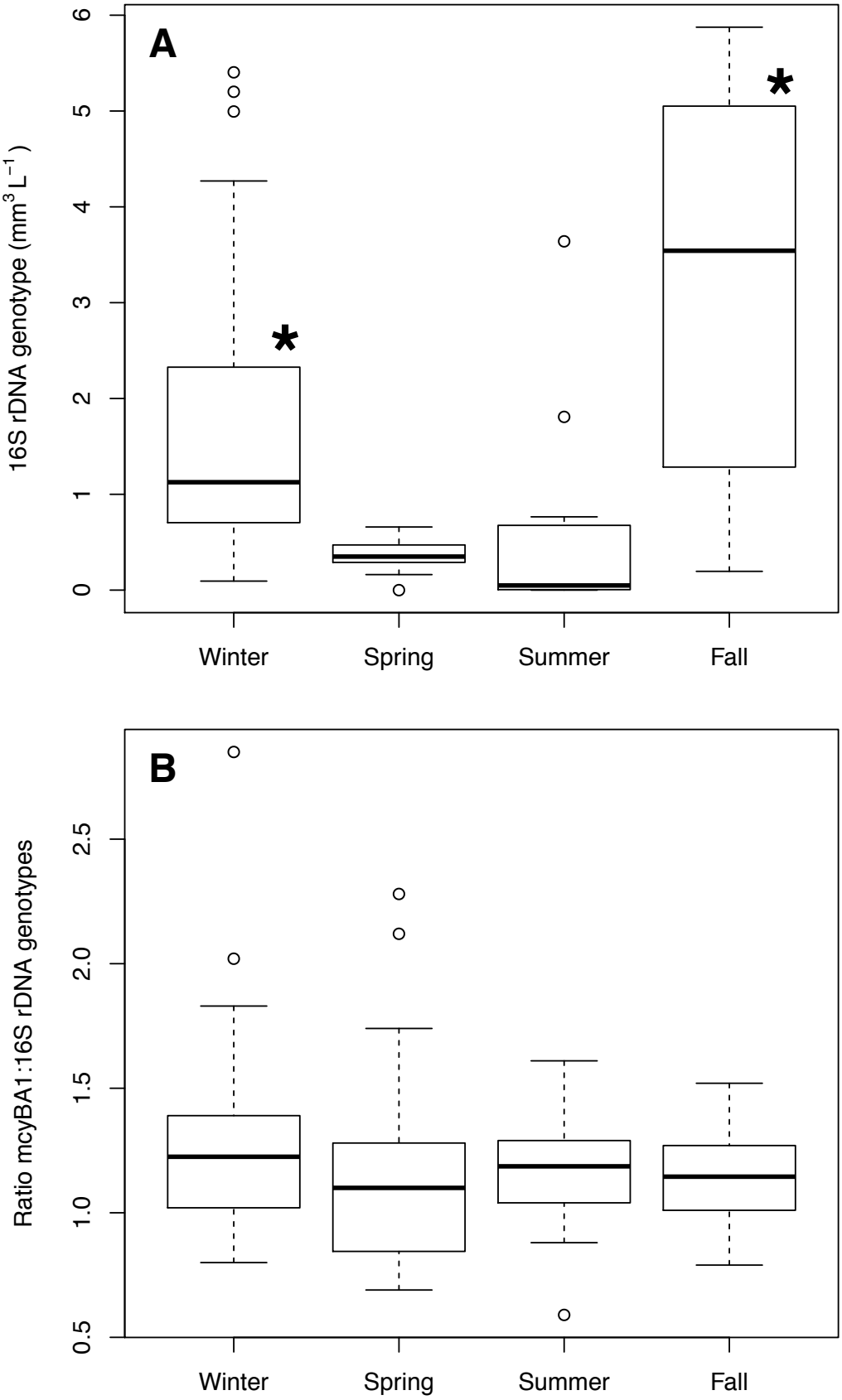

Figure 4. Garneau et al.

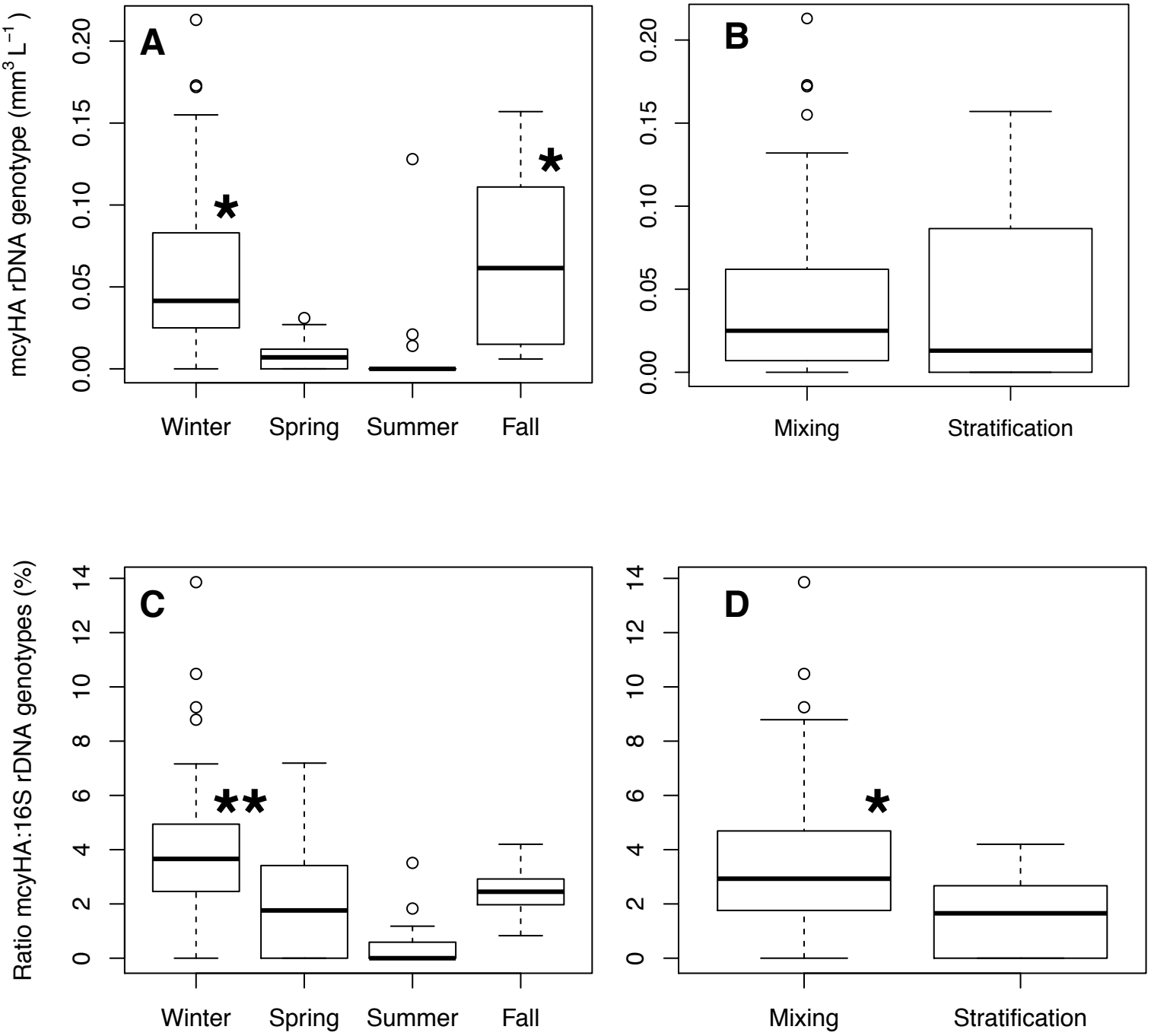

Figure 5. Garneau et al.